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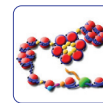
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Impact of decitabine on immunohistochemistry expression of the putative tumor suppressor genes FHIT, WWOX, FUS1 and PTEN in clinical tumor samples

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Abstract

Background: Since tumor suppressor gene function may be lost through hypermethylation, we assessed whether the demethylating agent decitabine could increase tumor suppressor gene expression clinically. For fragile histidine triad (*FHIT*), WW domain-containing oxidoreductase (*WWOX*), fused in sarcoma-1 (*FUS1*) and phosphatase and tensin homolog (*PTEN*), immunohistochemistry scores from pre- and post-decitabine tumor biopsies (25 patients) were correlated with methylation of the long interspersed nuclear element-1 (LINE-1) repetitive DNA element (as a surrogate for global DNA methylation) and with tumor regression.

Results: With negative staining pre-decitabine (score = 0), the number of patients converting to positive staining post-decitabine was 1 of 1 for *FHIT*, 3 of 6 for *WWOX*, 2 of 3 for *FUS1* and 1 of 10 for *PTEN*. In tumors with low pre-decitabine tumor suppressor gene scores (≤ 150), expression was higher post-treatment in 8 of 8 cases for *FHIT* ($P = 0.014$), 7 of 17 for *WWOX* ($P = 0.0547$), 7 of 12 for *FUS1* ($P = 0.0726$), and 1 of 16 for *PTEN* ($P = 0.2034$). If *FHIT*, *WWOX* and *FUS1* were considered together, median pre- versus post-decitabine scores were 60 versus 100 ($P = 0.0002$). Overall, tumor suppressor gene expression change did not correlate with LINE-1 demethylation, although tumors converting from negative to positive had a median decrease in LINE-1 methylation of 24%, compared to 6% in those not converting ($P = 0.069$). Five of 15 fully evaluable patients had reductions in tumor diameter (range 0.2% to 33.4%). Of these, three had simultaneous increases in three tumor suppressor genes (including the two patients with the greatest tumor regression) compared to 2 of 10 with tumor growth ($P = 0.25$).

Conclusions: In tumors with low tumor suppressor gene expression, decitabine may be associated with increased expression of the tumor suppressor genes *FHIT*, *FUS1*, and *WWOX*, but not *PTEN*.

Keywords: Decitabine, *FHIT*, *FUS1*, *WWOX*, *PTEN*, Tumor suppressor genes, LINE-1 methylation

Background

Several tumor suppressor genes have now been described, and tumor suppressor gene silencing by mutation, deletion, or hypermethylation [1,2] is an important component of tumorigenesis. Treatment of cell lines or xenograft-bearing animals with the demethylating agents decitabine and 5-azacytidine has been reported to increase

expression of a variety of tumor suppressor genes [3-6]. Decitabine may upregulate gene expression through both methylation-dependent and methylation-independent mechanisms [1,2].

Decitabine is active clinically in some hematologic malignancies [7]. Administration of low doses of decitabine daily for multiple successive days may be most effective against hematological malignancies, and is also particularly likely to induce DNA demethylation [7].

In patients with refractory malignancies receiving low dose decitabine on days 1 to 5 \pm days 8 to 12 each cycle,

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we biopsied tumors before day 1 and on day 12 of cycle 1, and found in patient tumors that decitabine decreased methylation of the long interspersed nuclear element-1 (LINE-1) repetitive DNA element (as a surrogate for global DNA methylation) [8], while it increased tumor expression of the copper transport protein-1 (CTR1, a copper/platinum transporter) [8], the Ras homolog gene family member A (RhoA, an endocytosis regulator) [9], and the reduced folate carrier-1 (RFC1, a folate transporter) [9]. In pre-decitabine tumor samples, expression of CTR1 [8] and RhoA [9] was lower and LINE-1 methylation tended to be higher in patients who were ≤ 3 months versus > 3 months beyond most recent prior therapy [8], and LINE-1 methylation correlated inversely with expression of CTR1 [8] and RhoA [9].

Based on our observations with CTR1 [8], LINE-1 [8], and RhoA [9], we then investigated whether expression of selected tumor suppressor genes would vary with time from last treatment, with LINE-1 methylation and with decitabine treatment. The tumor suppressor genes assessed were fragile histidine triad (*FHIT*), WW domain-containing oxidoreductase (*WWOX*), fused in sarcoma-1 (*FUS1*) and phosphatase and tensin homolog (*PTEN*).

FHIT is a proapoptotic tumor suppressor gene that encodes the fragile histidine triad protein (FHIT, also known as bis-(5'-adenosyl) triphosphatase), and *FHIT* inactivation or loss occurs in many tumor types [10]. Loss of FHIT leads to alterations in the DNA damage response checkpoint, resulting in increased DNA instability [11]. Loss of *FHIT* expression is commonly associated with hypermethylation of the gene, and frequent *FHIT* hypermethylation has been reported in hepatocellular carcinomas [12], and in carcinomas of the larynx [13], breast [14], lung [14], cervix [15], vulva [16], and kidney [17]. The demethylating agent decitabine may increase expression of FHIT in cancer cell lines [3].

WWOX is a large gene spanning the chromosomal fragile site 16D [18]. It encodes the protein WW domain-containing oxidoreductase (WWOX) which may play a role in apoptosis [19], cell metabolism [20], and modulation of the activity of multiple interacting transcription factors [21]. *WWOX* is generally strongly expressed in various normal tissues [22], but its expression by immunohistochemistry (IHC) is absent or weak in many cancers arising from tissues that generally express *WWOX*, including cancers of the breast [18], ovary [23], bladder [24], and esophagus [25], and in leukemias [19]. Exposure to carcinogens such as cigarette smoking extract can lead to downregulation of *WWOX* expression [24]. *WWOX* downregulation has been noted to occur via promoter methylation in various malignancies [25-27], and decitabine may restore *WWOX* expression [3,4].

The *FUS1* gene (also known as tumor suppressor candidate 2 or *TUSC2*) is located in the chromosomal

3p21.3 region. In lung cancers and various other malignancies, this chromosomal region is frequently deleted and *FUS1/TUSC2* expression is often lost [28]. *FUS1* functions as a tumor suppressor gene by inducing apoptosis through activation of the intrinsic mitochondrial-dependent and Apaf-1-associated pathways [28]. *FUS1/TUSC2* may be hypermethylated in cancers of the head and neck, and decitabine may reverse this hypermethylation [29]. In breast cancer cell lines, decitabine increased expression of *FUS1/TUSC2* despite lack of gene methylation [30], in keeping with the known ability of decitabine to increase gene expression through both methylation-dependent and methylation-independent mechanisms [1,2].

PTEN functions as a tumor suppressor gene by negatively regulating the Akt pathway, and it is one of the most frequently inactivated tumor suppressor genes in human cancers [31]. *PTEN* hypermethylation has been reported to be common in several tumor types [32-35]. Demethylating agents have been reported to restore *PTEN* expression in cell lines with hypermethylated *PTEN* [5,6]. However, *PTEN* protein expression did not change with decitabine exposure in ovarian cancer cell lines [36], and the role of promoter hypermethylation in silencing *PTEN* expression is not clear-cut. While the above studies suggested a role for *PTEN* promoter hypermethylation, several other studies across a range of malignancies have failed to detect significant *PTEN* promoter methylation [37-40]. It has been noted that the *PTENP1* pseudogene (that has 98% homology with *PTEN*) is frequently hypermethylated in tumors and cell lines, while *PTEN* is not, and reports of methylation of *PTEN* have been attributed by some authors to misinterpretations of hypermethylation of *PTENP1* [38].

Results

Patient characteristics

Patient characteristics are presented in more detail in an earlier publication on this patient group [8]. Patient numbers varied slightly between tumor suppressor genes since insufficient biopsy material was available for some assessments. Twenty-five of 31 patients who were initially entered on our decitabine phase I clinical trial [8] had sufficient tissue to permit at least one IHC assessment (either pre- or post-decitabine) of at least one of the four tumor suppressor genes of interest. These 25 patients included 14 males and 11 females, with a median (range) age of 53 (20 to 75) years. Tumor types included cancers of the breast (four patients), kidney (three), head and neck (three, including one adenocystic carcinoma), lung (one), stomach (one), and appendix (one), malignant melanomas (four), thymic neoplasms (three), neuroendocrine tumors (two), lymphomas (two), and desmoplastic tumor (one). Patients had received a median (range) of five (1 to 14)

prior systemic regimens and a median (range) of two (0 to 6) prior targeted agents.

Tumor suppressor gene immunohistochemistry scores versus time from last therapy

Comparing patients undergoing pre-decitabine tumor biopsy ≤ 3 months after last prior chemotherapy or targeted therapy to those undergoing initial biopsy > 3 months after last therapy are presented, PTEN scores were significantly higher in patients with longer time intervals since last treatment ($P = 0.007$), and there was a trend towards higher FUS1 scores in later biopsies ($P = 0.15$), while there was no association of FHIT and WWOX scores with time from last treatment.

Tumor suppressor gene immunohistochemistry scores versus LINE-1 methylation

Tumor suppressor gene scores did not correlate significantly with LINE-1 methylation in pre-decitabine tumor samples, nor in pre-and post-decitabine samples combined (Table 1). However, if pre-decitabine scores for FHIT, FUS1 and WWOX were considered together, tumors with IHC scores = 0 for one of these genes (eight observations) had significantly higher LINE-1 methylation than did tumors with IHC scores > 0 (58 observations) (median 61.6% versus 45.4%, $P = 0.0481$).

Decitabine effect on tumor suppressor gene immunohistochemistry scores

For tumors with initially low tumor suppressor gene IHC expression (scores ≤ 150), expression was higher post-decitabine than pre-decitabine for FHIT ($P = 0.0140$), with a trend to higher expression post-decitabine for WWOX ($P = 0.0547$) and FUS1 ($P = 0.0726$), and little effect for PTEN ($P = 0.2034$) (Table 2, Figure 1). Overall, for pre-decitabine scores of ≤ 150 , there was an increase in the score post-decitabine in 8 of 8 cases for FHIT, 7 of 17 for WWOX, 7 of 12 for FUS1, but only 1 of 16 for PTEN. The proportion of cases with a post-decitabine increase was significantly higher for FHIT, WWOX and FUS1 combined than for PTEN ($P = 0.003$ by Fisher's exact test). If FHIT, WWOX and FUS1 were considered together then, for cases with initial scores ≤ 150 , the median

scores pre- and post-decitabine were 60 and 100, respectively ($P = 0.0002$).

Of those tumors that initially stained negatively for a tumor suppressor gene (pre-decitabine score = 0), there was conversion to positive staining post-decitabine for FHIT in 1 of 1 patients, for WWOX in 3 of 6 patients, for FUS1 in 2 of 3 patients, and for PTEN in 1 of 10 patients.

Post-decitabine changes in tumor suppressor gene immunohistochemistry scores versus percentage changes in LINE-1 DNA methylation

While there was a weak negative correlation between percentage change in LINE-1 methylation and IHC score for each tumor suppressor gene, this did not achieve significance (Table 3). If we only considered tumors that initially stained negatively for a gene, there was a stronger trend ($P = 0.069$) for ones that converted from negative to positive staining with decitabine to have a greater decrease in LINE-1 methylation with decitabine (median decrease of 24% in LINE-1 methylation) than for those that did not convert (median decrease in LINE-1 methylation of 6%).

Tumor suppressor gene immunohistochemistry score changes and tumor regression

Of the 25 patients included, both change in tumor size with the first cycle of therapy and change in IHC scores for all four tumor suppressor genes could be assessed in 15 patients. Of these, 10 patients had immediate tumor growth, while five patients had a reduction in tumor diameter of 0.2%, 2%, 4.7%, 22.1% and 33.4%, respectively. Among the five with any degree of tumor regression, all five had increases in FHIT scores, three had increases in WWOX scores, three had increases in FUS1 scores, and one had an increase in PTEN score. All five had an increase in at least one tumor suppressor gene, and three had simultaneous increases in three tumor suppressor genes (including the two patients with the greatest tumor regression), compared to 2 of 10 fully evaluable patients with tumor growth who had an increase in expression of three tumor suppressor genes ($P = 0.25$ by Fisher's exact test). Median (range) number of tumor suppressor genes increasing with decitabine was three (1 to 3) in those with tumor regression versus two (0 to 3) in fully evaluable patients with tumor growth ($P = 0.34$).

Discussion

In our phase I trial of the demethylating agent decitabine we had previously reported that percentage LINE-1 DNA methylation correlated inversely with expression of the copper/platinum transporter CTR1 and the endocytosis regulator RhoA, and that decitabine treatment

Table 1 Correlation of tumor suppressor gene encoded proteins with LINE-1 methylation (pre- and post-decitabine)

Protein	n	Spearman r	P
FHIT	41	-0.23	0.15
WWOX	44	0.09	0.57
FUS1	41	0.15	0.35
PTEN	40	0.03	0.83

FHIT, fragile histidine triad; FUS1, fused in sarcoma-1; LINE-1, long interspersed nuclear element-1; PTEN, phosphatase and tensin homolog; WWOX, WW domain-containing oxidoreductase.

Table 2 Median tumor suppressor gene encoded protein scores post- versus pre-decitabine for patients with pre-decitabine scores (≤ 150), and number of patients with an increase, no change or decrease in scores with decitabine

Protein	n	Score pre-decitabine	Score post-decitabine	P	Number with increase in score	Number with no change in score	Number with decrease in score
FHIT	8	100	175	0.014	8	0	0
WWOX	17	30	100	0.05	7	8	2
FUS1	12	67.5	100	0.07	7	2	3
PTEN	16	0	0	0.20	1	10	5
FHIT, WWOX or FUS1	37	60	100	0.0002	22	10	5

FHIT, fragile histidine triad; FUS1, fused in sarcoma-1; PTEN, phosphatase and tensin homolog; WWOX, WW domain-containing oxidoreductase.

was associated with an increase in expression of CTR1, RhoA and of the folate carrier RFC1 in patients in whom expression was initially low [8,9]. Early phase clinical trials also suggest that addition of decitabine to a platinum agent may partially reverse platinum-resistance in advanced ovarian carcinomas [41-43]. Hence, there is interest in further exploring the role of decitabine and other demethylating agents as resistance-modulating therapies.

In this further assessment of patients included in our phase I trial of low-dose single-agent decitabine, we assessed the impact of decitabine on expression of selected tumor suppressor genes. Loss of tumor suppressor gene function through gene deletion, mutation, or silencing (through promoter hypermethylation or other means) is

important in tumorigenesis, but replacing lost tumor suppressor gene function is difficult. Attempts at tumor suppressor gene replacement by adenovirus-mediated gene therapy have met with modest early indications of success [44-46], although administration has generally been by local injection or similar means, and systemic delivery to widespread metastases is challenging. However, it has recently been demonstrated that intravenous administration of the tumor suppressor gene FUS1/TUSC2 in DOTAP nanoparticles is capable of systemic delivery to disseminated disease [47], and this approach is undergoing further investigation.

Our results with decitabine suggest that demethylating agents may be capable of upregulating expression of

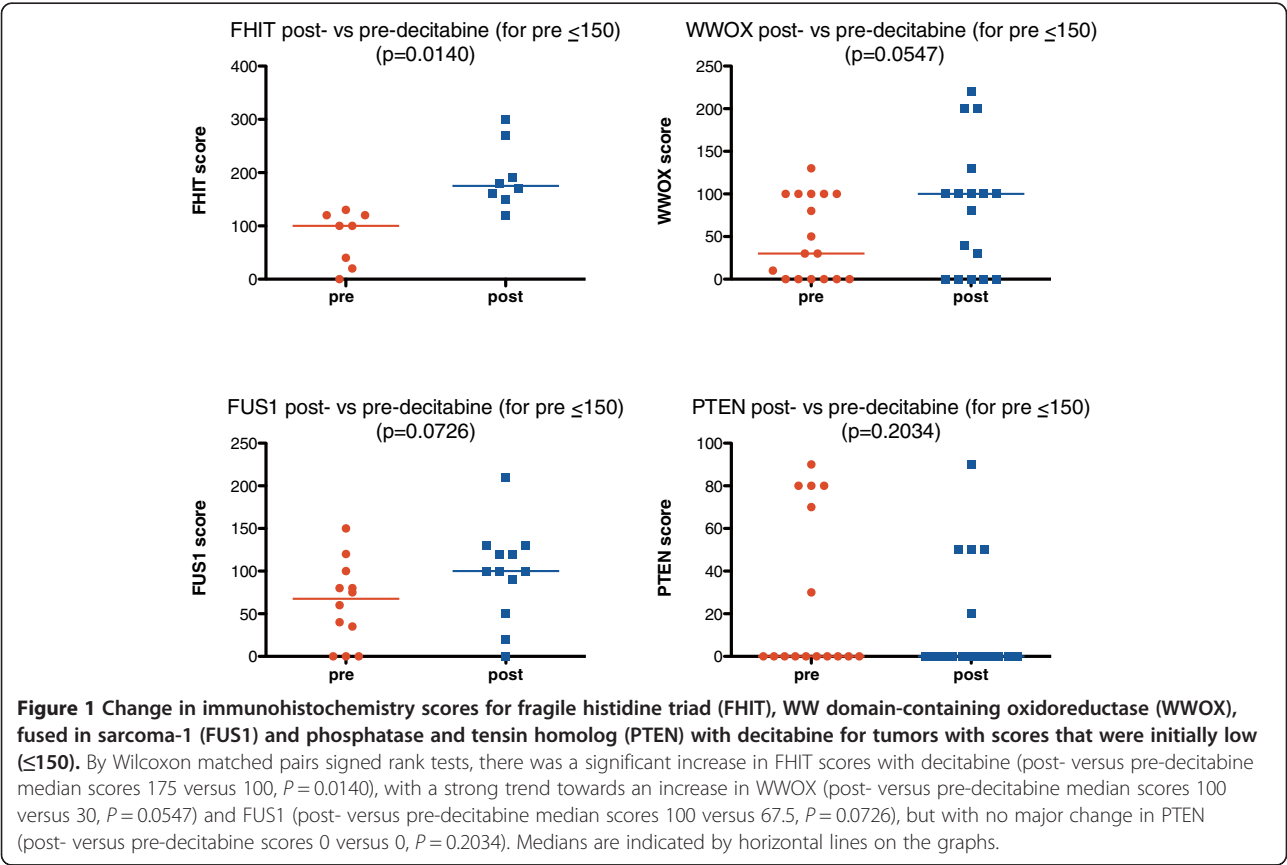


Table 3 Change in tumor suppressor gene encoded protein score versus percentage change in LINE-1 methylation with decitabine

Protein	n	Spearman r	P
FHIT	16	-0.34	0.19
WWOX	19	-0.04	0.87
FUS1	16	-0.03	0.91
PTEN	15	-0.26	0.36
Any tumor suppressor gene	67	-0.11	0.39

FHIT, fragile histidine triad; FUS1, fused in sarcoma-1; PTEN, phosphatase and tensin homolog; WWOX, WW domain-containing oxidoreductase.

selected tumor suppressor genes in some patients. Low patient numbers have limited statistical power but, despite this, there was a statistically significant increase in expression of FHIT following decitabine, with a strong statistical trend towards increase in expression of WWOX and FUS1, and combining data for FHIT, WWOX and FUS1 indicated a significant increase in IHC expression of these three tumor suppressor genes together. This is sufficient to encourage further assessment of the ability of demethylating agents to restore tumor suppressor gene function in situations where it has been decreased or lost due to promoter hypermethylation. For each of these tumor suppressor genes, available published data suggest that promoter methylation may be an important cause of loss of gene function [3,4,12-17,25-27,29,48,49], although we did not have sufficient residual tissue to permit us to assess this in our study, and it is probable that other mechanisms such as gene deletion or mutation were responsible for low expression in a proportion of the tumors. We also did not have sufficient tissue available to assess whether increase in tumor suppressor gene protein expression with decitabine was associated with reduction in tumor suppressor gene DNA methylation.

There was no apparent increase in PTEN IHC scores with decitabine treatment, and changes in PTEN scores were significantly lower than changes in scores for the other tumor suppressor genes. This is in keeping with the indication from many publications that promoter methylation may not be an important cause of loss of PTEN expression [37-39], and in keeping with the hypothesis that an apparent role for PTEN promoter methylation may be due to misinterpretation of data arising from methylation of the closely related pseudo-gene PTENP1 [38]. An additional potential explanation for the lack of impact of decitabine on PTEN is that, for at least some genes, gene reactivation in response to decitabine requires chromatin remodeling in addition to DNA demethylation if histone modification is playing a role in gene silencing [50].

All five patients experiencing tumor regression with decitabine had an increase in the IHC score of at least

one tumor suppressor gene, and three (including the two with the greatest regression) had an increase in IHC scores of three tumor suppressor genes, compared to 2 of 10 fully evaluable patients who had tumor growth who had an increase in IHC scores for three tumor suppressor genes, and the median number of tumor suppressor genes that increased with decitabine was three in patients with tumor regression versus two in patients with tumor growth. Further assessment will be needed to determine if change in tumor suppressor genes with decitabine impacts the probability of achieving tumor regression.

Conclusions

Overall, our data add further evidence that exploration of demethylating agents in solid tumors may be of interest. It may be of particular interest to explore them in tumors that are demonstrated to have low expression of tumor suppressor genes in association with promoter methylation.

Methods

This study was approved by the MD Anderson Cancer Center Research Ethics Board, and recruited consenting patients with advanced malignancies, tumors that could be safely biopsied, and organ function meeting eligibility requirements [8]. Decitabine was supplied under a Collaborative and Research Development Agreement by the National Cancer Institute Division of Cancer Treatment and Diagnosis. Decitabine doses of 2.5, 5, or 10 mg/m²/day on days 1 to 5 and 8 to 12 each 4-week cycle or 15 or 20 mg/m²/day on days 1 to 5 each cycle were administered over 1 hour, with filgrastim added at higher doses [8].

Tumor biopsies were performed pre-decitabine and on day 12, cycle 1 [8]. Formalin-fixed, paraffin-embedded tissue sections (5 µm thick) were deparaffinized in Xylene (10 minutes × 3), followed by hydration in sequenced graded alcohols (5 minutes each). Heat-induced epitope retrieval was performed in DAKO solution for 30 minutes at 121°C, followed by 10 minutes at 90°C using a Decloaking chamber (Biocare, Concord, CA), followed by a 30-minute cool-down. Prior to antibody immunostaining, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 minutes. To block non-specific antibody binding, tissues were incubated in 10% fetal bovine serum/Tris-buffered saline with Tween 20 for 30 minutes. Primary incubation antibodies are presented in Table 4. This was followed by incubation with Envision plus labeled polymer, anti-rabbit-horseradish peroxidase antibody (DAKO, Carpinteria, CA) for 30 minutes at room temperature. The FUS1 protocol was as previously described [47]. Time monitoring staining development was performed with diaminobenzidine, using a reliable positive control sample.

Table 4 Antibodies used for immunohistochemistry

Protein	Source	Dilution
FHIT	Thermo Scientific, Waltham, MA, USA	1:100
WVVOX	Abcam, Cambridge, MA, USA	1:100
FUS1	Homemade, rabbit polyclonal	1:400
PTEN	Cell Signaling, Danvers, MA, USA	1:75

FHIT, fragile histidine triad; FUS1, fused in sarcoma-1; PTEN, phosphatase and tensin homolog; WVVOX, WW domain-containing oxidoreductase.

Slides were counterstained with hematoxylin, dehydrated, cleared and mounted.

Participating pathologist MIN scored staining intensity as 0 to 3+ and generated IHC scores of 0 to 300 by multiplying the percent of tumor cells staining by the staining intensity. For PTEN, we assessed both cytoplasmic and nuclear staining scores, but report only on the cytoplasmic scores since results were similar for the two staining sites. Change in IHC score was defined as the day 12 score minus the day 1 score.

As previously reported, the LINE-1 assay was used to define percentage of DNA CpG islands that were methylated, as a surrogate for global DNA methylation [8,51]. Change in LINE-1 methylation was defined as the day 12 value minus the day 1 value divided by the day 1 value.

While low patient numbers limited statistical power, GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used to assess non-parametric two-tailed statistics (Spearman tests for correlations, Wilcoxon signed rank tests for paired comparisons, and Mann-Whitney tests and Fisher exact tests for comparisons of two groups).

Abbreviations

CTR1: copper transport protein-1; FHIT: fragile histidine triad; FUS1: fused in sarcoma-1; IHC: immunohistochemistry; LINE-1: long interspersed nuclear element-1; PTEN: phosphatase and tensin homolog; PTENP1: phosphatase and tensin homolog pseudogene; RFC1: reduced folate carrier-1; RhoA: Ras homolog gene family member A; TUSC2: tumor suppressor candidate 2; WVVOX: WW domain-containing oxidoreductase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DJS designed and oversaw the overall study, analyzed the data and drafted the manuscript. MIN performed all immunohistochemistry on tumor samples. JJ assessed LINE-1 methylation of tumor samples. DH contributed to patient recruitment. SG oversaw tumor biopsies. MA contributed to WVVOX studies. JPI oversaw LINE-1 methylation studies. RK oversaw patient recruitment. IIV oversaw tumor sample collection, storage, retrieval and immunohistochemistry. All authors read and approved the final manuscript.

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